REMARKS

Claims 1-53 are pending in this application. Claims 29-53 are withdrawn. Reconsideration is respectfully requested in view of the following remarks.

I. Claim Rejections Under 35 U.S.C. 102(b) to Schantz et al.:

The Examiner rejected claims 16, 17, 21 and 27 under 35 U.S.C. 102(b) as allegedly anticipated by Schantz et al. (J. AOAC 61:96-99 (1978). It is believed that this rejection is not applicable in view of the amendments made to the claims in Applicants Response, dated March 9, 2005.

The Examiner states that Schantz teaches a solvent composition in buffer having a pH of 4.2. See Office Action, p. 10. As discussed during the telephonic interview, Applicants respectfully submit that Schantz does not disclose, teach or suggest a composition as provided in the amended claims. The amended claims now recite "a pharmaceutically acceptable buffered saline capable of providing a buffered pH range between pH 5 and ph 6 +/- 10%." As Applicants discussed with the Examiner, a pH of 4.2, as was disclosed in Schantz, is not within 10% of the recited limitation of pH 5-6. The pH of a solution is calculated as the negative logarithmic value of the hydrogen ions present in a solution. *See* Lehninger, A.L., Biochemistry, 2nd ed. (1978), pp. 45-50 (attached herein as Exhibit B). As stated in Lehninger "[n]ote that the pH scale is logarithmic, not arithmetic. To say that two solutions differ in pH by 1 pH unit means only that one solution has 10 times the hydrogen-ion concentration of the other." *Id.* at p. 46. In other words, a difference of 1 pH unit is equivalent to 1000% more hydrogen ions being present in the solution. Because of this logarithmic scale, a pH of 4.2 has approximately 640% more H^+ ions than a solution at pH 5.0, the lower value of the claimed scale limitation. Because Schantz is not within the +/- 10% limitation now claimed, Schantz does not anticipate the presently claimed compositions.

In light of the arguments presented above and the amended claims, Applicants respectfully request that the Examiner withdraw the rejection.

II. Claim Rejection Under 35 U.S.C. 112, First Paragraph:

The Examiner rejected claims 1-28 under 35 U.S.C. 112, first paragraph, as being allegedly non-enabling for the claimed invention. Applicants believe that this rejection is not applicable in view of the following comments and the attached Declaration of Dr. Moyer ("Moyer Decl.," attached herein as Exhibit C).

During the telephonic interview, Applicants discussed with the Examiner the state of the art as it pertains to botulinum toxin purification and buffer art at the time of filing of the instant application. Applicants now provide the Examiner with a Declaration from the named inventor, Dr. Elizabeth Moyer, in support of the Applicant's arguments pertaining to the Office Action.

Dr. Moyer first describes the necessity of column-purified formulations in order to achieve the stability claimed in the instant application. *See* Moyer Decl. at ¶ 2. In contrast to the prior art cited in the Office Action, dated June 1, 2004, the state of the art was beyond the precipitation purified botulinum toxins that are represented in the cited references. *See* Boroff et al. (pg. 1-62, in "Microbial Toxins" S Kadis, T. Montie and S. Ajl, eds., Academic Press, 1971); Gartlan et al. (Otolar-Head & Neck Surg. 108:135-140 (1993)); and Schantz & Johnson (In "Therapy with Botulinum Toxin" Jankovic et al. eds., Marcel Dekker, Inc., New York, 41-51 (1994)), cited in the Office Action, dated June 1, 2004). The Boroff article, published in 1971, is a review of the state of the art in the 50's and 60's. The article presents work done on partially purified extracts of botulinum toxin preparations (see pgs. 9-15). Similarly, the Gartlan and Schantz & Johnson articles also presents work on non-column chromatographed botulinum toxin preparations.

In contrast, Dr. Moyer in her declaration provides evidence that the state of the art of botulinum toxin purification at the time of filing was column chromatography purification of the botulinum toxin after the partial purification of the bacterial or culture extracts, and that column chromatography could be used to purify each botulinum toxin serotype claimed herein. *See* Moyer Decl. at ¶¶ 2-3. Therefore, Dr. Moyer's Declaration provides evidence as to what one of ordinary skill in the art would know in connection with botulinum toxin purification, as taught and disclosed in the instant application.

In light of the evidence provided by Dr. Moyer in her Declaration, Applicants believe that the cited articles are not relevant to the state of the prior art at the time of filing, and does not support the Examiner's contention that the prior art does not support a stable pharmaceutical formulation of botulinum toxin as claimed. Applicants respectfully request that this rejection be withdrawn.

Dr. Moyer also describes the state of the art of botulinum toxin functionality, as well as shared characteristics between the various botulinum toxin serotypes, demonstrating that one of skill in the art at the time of filing would understand that the botulinum toxin serotypes were identical in functionality in connection with their molecular target. *See* Moyer Decl. at ¶ 4. As further evidence that one of ordinary skill in the art could use any of the botulinum toxin serotypes in the pharmaceutical formulation claimed, Dr. Moyer describes that the botulinum toxins are well documented as being matched in physical and functional characteristics. Botulinum toxin serotypes A, B, C_1, C_2, D, E, F and G are also similar in terms of physical characteristics. This is evidenced in the use of ammonium sulfate precipitation, followed by column chromatography purification for each of the botulinum toxin serotypes. *See* Moyer Decl. at ¶ 3.

Furthermore, one of ordinary skill in the art would have known of the similar characteristics of the botulinum toxins as evidenced from their interchangeability in the treatment of various neuromuscular disorders. For example, in U.S. Patent No. 6,861,058, filed August 5, 2000, which is a divisional application to U.S. Patent Application Ser. No. 08/627,118, filed April 3, 1996, patentees state that although some botulinum toxins are preferred over others in regards to their efficacy and duration of effect (*see, e.g.,* botulinum type F preferred in sports injuries due to short duration of activity; '058 patent, col. 5, lines 4-6), in the treatment of excessive lacrimation due to cholinergic secretion "[a]ny serotype toxin alone or in combination could be used in this indication." '058 patent, col. 7, line 63 to col. 8, line 4. As further evidence, there are many more patents claiming the interchangeability of botulinum toxins in the treatment of a variety of neuromuscular diseases. By way of example only, *see, e.g.,* U.S. Patent Nos. 6,113,915; 6,139,845; 6,350,455; 6,358,513; all attached herein as Exhibit D. Therefore, one of ordinary skill in the art would have recognized that the botulinum toxins are

interchangeable in regards to treatment of various disorders, owing to their closely similar characteristics that allow this interchangeability to occur.

In her Declaration, Dr. Moyer also provides supporting evidence that one of ordinary skill in the art would look to pharmaceutically acceptable agents capable of buffering in the pH range claimed. *See* Moyer Decl. at ¶ 5. For example, Dr. Moyer describes the nature of buffering agents, and the guidance provided by the instant specification to allow one of ordinary skill in the art to use a pharmaceutically acceptable agent capable of buffering in the claimed ranges. Therefore, contrary to the Examiner's assertions, the specification provides adequate guidance to enable one of ordinary skill in the art to practice the claimed invention.

In summary, Applicants believe that they have provided extensive teaching regarding the purification of a wide range of the serotypes of botulinum toxin, and preparing then in a formulation that is stable for extended periods of time. Purification of types A and B are described in detail in section III of the specification beginning at about page 10, line 22. As noted at page 12, line 22 and in the Declaration attached, botulinum toxin types C_1, C_2, D, E, F or G may be prepared and purified according to methods known in the art. One skilled in the art would understand how to adapt the disclosed methods to provide stable formulations comprising types A and C-G from reading the specification and references available prior to the filing of this application.

Based on the amendment to the claims and the remarks above, Applicants respectfully request withdrawal of this rejection.

CONCLUSION

In light of the remarks set forth above, Applicant believes that the claims are in condition for allowance. Applicant respectfully solicits the Examiner to expedite the prosecution of this patent application to issuance. Should the Examiner have any questions, the Examiner is encouraged to telephone the undersigned.

Respectfully submitted,

Date: April 15, 2005 By: Albert P. Halluin

Albert P. Halluin
Registration No. 25,227

Lorelei Westin
Registration No. 52,353

650 Page Mill Road
Palo Alto, California 94304-1050
(650) 565-3585
Customer No. 021971

PENDING CLAIMS

1. (Pending) A stable liquid pharmaceutical botulinum toxin formulation, comprising

a pharmaceutically acceptable buffered saline capable of providing a buffered pH range between pH 5 and pH 6 $\pm$ 10%, and

purified botulinum toxin;

wherein said formulation is stable as a liquid for at least one year at a temperature between 0 and 10 degrees centigrade $\pm$ 10%.

2. (Pending) The formulation of claim 1, wherein said temperature is 5$\pm$3 degrees centigrade.

3. (Pending) The formulation of claim 1, wherein said temperature is 4$\pm$2 degrees centigrade.

4. (Pending) The formulation of claim 1, wherein said buffered pH range is pH 5.6$\pm$ 10%.

5. (Pending) The formulation of claim 1, wherein said toxin formulation is stable in liquid form for at least two years.

6. (Pending) The formulation of claim 1, wherein said buffer has a pK in the range of pH 4.5-6.5.

7. (Pending) The formulation of claim 6, wherein said buffer is selected from the group consisting of phosphate buffer, phosphate-citrate buffer, and succinate buffer.

8. (Pending) The formulation of claim 1, wherein said botulinum toxin is of a botulinum toxin type selected from the group consisting of Types A, B, C_1, C_2, D, E, F and G.

9. (Pending) The formulation of claim 8, wherein said botulinum toxin is botulinum toxin Type B present at a concentration in the range of 100-20,000 U/ml ± 10%.

10. (Pending) The formulation of claim 9, wherein said botulinum toxin Type B is present in a high molecular weight complex of 700 kilodaltons (kD) ± 10%.

11. (Pending) The formulation of claim 9, wherein said botulinum toxin Type B is present at a concentration between 1000-5000 U/ml.

12. (Pending) The formulation of claim 8, wherein said botulinum toxin is botulinum toxin Type A, present at a concentration in the range of between 20-2000 U/ml.

13. (Pending) The formulation of claim 12, wherein said botulinum toxin Type A is present at a concentration in the range of between 100-1000 U/ml.

14. (Pending) The formulation of claim 1, which further includes an excipient protein.

15. (Pending) The formulation of claim 14, wherein said excipient protein is selected from the group consisting of serum albumin, recombinant human serum albumin, and gelatin.

16. (Pending) A stable liquid pharmaceutical botulinum toxin formulation, comprising

a pharmaceutically acceptable liquid buffered saline capable of providing a buffered pH range between pH 5 and pH 6 ± 10%, and

purified botulinum toxin;

wherein said toxin formulation is stable as a liquid for at least about 6 months at a temperature between 10 and 30 degrees centigrade ± 10%.

17. (Pending) The formulation of claim 16, wherein said temperature is 25°C ± 10%.

18. (Pending) The formulation of claim 16, wherein said buffered pH range is pH 5.6.± 10%.

19. (Pending) The formulation of claim 16, wherein said buffer has a pK in the range of pH between 4.5-6.5.

20. (Pending) The formulation of claim 19, wherein said buffer is selected from the group consisting of phosphate buffer, phosphate-citrate buffer, and succinate buffer.

21. (Pending) The formulation of claim 16, wherein said botulinum toxin is of a botulinum toxin type selected from the group consisting of Types A, B, C_1, C_2, D, E, F and G.

22. (Pending) The formulation of claim 21, wherein said botulinum toxin is botulinum toxin Type B present at a concentration of between 100-20,000 U/ml $\pm$ 10%.

23. (Pending) The formulation of claim 22, wherein said botulinum toxin Type B is present in a high molecular weight complex of 700 kD $\pm$ 10%.

24. (Pending) The formulation of claim 22, wherein said botulinum toxin Type B is present at a concentration in the range of between 1000-5000 U/ml.

25. ((Pending) The formulation of claim 21, wherein said botulinum toxin is botulinum toxin Type A, present at a concentration in the range of between 20-2000 U/ml.

26. (Pending) The formulation of claim 25, wherein said botulinum toxin is botulinum toxin Type A, present at a concentration in the range of between 100-1000 U/ml.

27. (Pending) The formulation of claim 16, which further includes an excipient protein.

28. (Pending) The formulation of claim 25, wherein said excipient protein is selected from the group consisting of serum albumin, human serum albumin, and gelatin.

29. (Withdrawn) A method of treating a patient in need of inhibition of cholinergic input to a selected muscle, muscle group, gland or organ, comprising administering to the selected muscle, muscle group, gland or organ of the patient a pharmaceutically effective dose of liquid botulinum toxin formulation which includes a pharmaceutically acceptable buffer capable of providing a buffered pH range between about pH 5 and pH 6, and

isolated botulinum toxin;

wherein said toxin formulation is stable as a liquid for at least one year at a temperature between about 0 and 10 degrees centigrade or for at least six months at a temperature between about 10 and 30 degrees centigrade.

30. (Withdrawn) The method of claim 29, wherein said patient is suffering from a disorder selected from the group consisting of spasticity, blepharospasm, strabismus, hemifacial spasm, dystonia, otitis media, spastic colitis, animus, urinary detrusor-sphincter dyssynergia, jaw-clenching, and curvature of the spine.

31. (Withdrawn) The method of claim 30, wherein said patient is suffering from spasticity due to one or more of the group consisting of stroke, spinal cord injury, closed head trauma, cerebral palsy, multiple sclerosis, and Parkinson's disease.

32. (Withdrawn) The method of claim 30, wherein said patient is suffering from a dystonia selected from the group consisting of spasmodic torticollis (cervical dystonia), spasmodic dyshponia, limb distonia, laryngeal dystonia, and oromandibular (Meige's) dystonia.

33. (Withdrawn) The method of claim 29, wherein said selected muscle or muscle group produces a wrinkle or a furrowed brow.

34. (Withdrawn) The method of claim 29, wherein said muscle is a perineal muscle and wherein said patient is in the process of giving birth to a child.

35. (Withdrawn) The method of claim 29, wherein said patient is suffering from a condition selected from the group consisting of myofascial pain, headache associated with migraine, vascular disturbances, neuralgia, neuropathy, arthritis pain, back pain, hyperhydrosis, rhinnorhea, asthma, excessive salivation, and excessive stomach acid secretion.

36. (Withdrawn) The method of claim 29, wherein said formulation is stable as a liquid for at least one year at a temperature of about 5±3 degrees centigrade.

37. (Withdrawn) The method of claim 29, wherein said formulation is stable as a liquid for at least one year at a temperature of about 4±2 degrees centigrade.

38. (Withdrawn) The method of claim 29, wherein said formulation is stable as a liquid for at least two years at a temperature between about 0 and 20 degrees centigrade.

39. (Withdrawn) The method of a claim 29, wherein said buffered pH range is about pH 5.6±0.2

40. (Withdrawn) The method of claim 29, wherein said buffer has a pK in the range of pH 4.5-6.5.

41. (Withdrawn) The method of claim 29, wherein said buffer is selected from the group consisting of phosphate buffer, phosphate-citrate buffer, and succinate buffer.

42. (Withdrawn) The method of claim 29, wherein said botulinum toxin is a botulinum toxin serotype selected from the group consisting of serotypes A, B, C_1, C_2, D, E, F and G.

43. (Withdrawn) The method of claim 42, wherein said botulinum toxin is botulinum toxin Type B present at a concentration in the range of about 100-20,000 U/ml.

44. (Withdrawn) The method of claim 43, wherein said botulinum toxin Type B is present in a high molecular weight complex of about 700 kD.

45. (Withdrawn) The method of claim 43, wherein said botulinum toxin Type B is present at a concentration of about 1000-5000 U/ml.

46. (Withdrawn) The method of claim 42, wherein said botulinum toxin is botulinum toxin Type A, present at a concentration in the range of about 20-2000 U/ml.

47. (Withdrawn) The method of claim 46, wherein said botulinum toxin Type A is present at a concentration in the range of about 100-1000 U/ml.

48. (Withdrawn) The method of claim 29, which further includes an excipient protein.

49. (Withdrawn) The method of claim 48, wherein said excipient protein is selected from the group consisting of serum albumin, recombinant human serum albumin, and gelatin.

50. (Withdrawn) The method of claim 29, wherein said patient is refractory to botulinum toxin Type A and said botulinum toxin in said formulation is selected from the group consisting of botulinum serotypes B, C_1, C_2, D, E, F and G.

51. (Withdrawn) The method of claim 50, wherein said botulinum toxin in said formulation is botulinum toxin Type B.

52. (Withdrawn) The method of claim 29, wherein said patient is refractory to botulinum toxin Type B and said botulinum toxin in said formulation is selected from the group consisting of botulinum serotypes A, C_1, C_2, D, E, F and G.

53. (Withdrawn) The method of claim 52, wherein said botulinum toxin in said formulation is botulinum toxin Type A.

Table of prior art documents cited against European Patent No. 1 112 082, in the name of Elan Pharmaceuticals Inc.

New Reference	Opponents Reference	Prior Art
D1	E1, D5	M.C. Goodnough et al, Applied and Environmental Biology (1992), 58 (10), 3426-28
D2	E2	E.J. Schantz et al, article entitled "Quality of botulinum toxin for human treatment", Botulinum and Tetanus Neurotoxins (1993), 657-659
D3	E3	P. Hambleton and A.M. Pickett, article entitled "Potency equivalence of botulinum toxin preparations", Journal of the Royal Society of Medicine (1994), 87, 719
D4	E4	Thesaurus Vidal-Semp (CD ROM), 1/3 2003, Dysport
D5	E5	Thesaurus Vidal-Semp (CD ROM), 1/3 2003, Botox
D6	E6	Thesaurus Vidal-Semp (CD ROM), 1/3 2003, Neurobloc
D7	E7	P. Seller, article entitled "The biochemistry of botulinum toxin type B", Neurobiology (2000)
D8	E8	WO 93/05800
D9	E9	Tsui-J-K, Can. J. Neurol. Sci. (1985), 12, 314-316
D10	E10	WO 95/30431
D11	E11, D14	WO 95/17904
D12	D1	US 5,696,077
D13	D2	WO 94/00481
D14	D3	EP 0 593 176
D15	D4	US 5,512,547
D16	D6	Commercial product "Dysport" (approval by MGA in the UK in 1990), as evidenced by internet print-out from the website of its supplier, Ipsen Ltd.
D17	D7	McCellan et al, Toxicon (1996), 34 (9), 975-85
D18	D8	E.J. Schantz and E.A. Johnson, article entitled "Properties and use of botulinum toxin and other microbial neurotoxins in medicine", Microbiological Reviews, 56(1), 80-99, (1992).
D19	D9	E.J. Schantz and E.A. Johnson, article entitled "Preparation and Characterization of Botulinum Toxin Type A for Human Treatment", Therapy with Botulinum Toxin, ed. J. Jankovic and M.Hallett, Marcel Decker Inc. 1994, pp. 41-49
D20	D10	Doweiko, J.P. et al, article entitled "Role of Albumin in Human Physiology and Pathophysiology", Journal of Parenteral and Enteral Nutrition, 15, 1991, 207-211
D21	D11	US 5,562,899
D22	D12	US 5,714,468
D23	D13	WO 95/03041

D24	D15	Asher, B., J. Med. Esth. Et Chir. Derm. XXIII, 1996, 159-166
D25	D16	US 5,766,605
D26	D17	US 5,183,462

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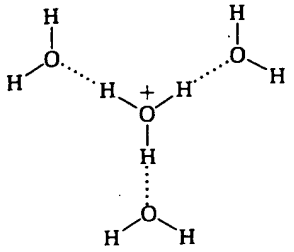
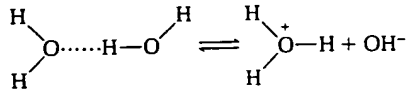
Figure 2-6
Hydrated form of hydronium ion ($H_9O_4^+$). The hydration shell is stable to 100°C.



bonded, provided that the internal energy of each molecule is favorable:



In this reaction two ions are produced, the hydronium ion (H_3O^+) and the hydroxide ion (OH^-). In a liter of pure water at 25°C at any given time there is only 1.0×10^{-7} mol of H_3O^+ ions and an equal amount of OH^- ions, as shown by electrical-conductivity measurements.

Although it has become the convention, for brevity, to use the symbol H^+ to designate the hydronium ion, it must be strongly emphasized that protons or hydrogen ions do not exist "bare" in water to any significant extent; they occur only in hydrated form. Moreover, the H_3O^+ or hydronium ion is itself further hydrated through additional hydrogen bonding with water to form the $H_9O_4^+$ ion, as well as more highly hydrated forms (Figure 2-6). The hydroxide ion is also hydrated in liquid water.

Table 2-4 shows that the apparent rate of migration of H_3O^+ ions in an electric field is many times greater than that of the univalent cations Na^+ and K^+. This anomaly results because a proton can jump very rapidly from a hydronium ion to a neighboring water molecule to which it is hydrogen-bonded. Thus a positive electric charge can move a given distance from one molecule of water to another with little movement of the water molecules themselves. A series of such proton jumps has the effect of translocating protons at a rate that is much higher than the rate of diffusive or bulk movement of H_3O^+ ions per se (Figure 2-7). Proton jumps along immobilized water molecules in the crystal lattice of ice are responsible for the fact that ice, despite its rigid structure, has about the same electrical conductivity as liquid water. Conduction of protons through hydrogen-bonded water molecules, called tunneling, may be an important phenomenon in biological systems.

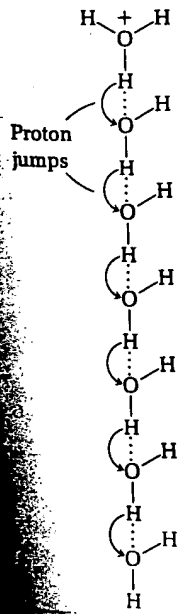
Table 2-4 Electrical mobility of some cations at infinite dilution (25°C)

Ion	Mobility, $cm^2\ V^{-1}\ s^{-1}$
H^+	36.3×10^{-4}
Na^+	5.2×10^{-4}
K^+	7.6×10^{-4}
NH_4^+	7.6×10^{-4}
Mg^{2+}	5.4×10^{-4}
Li^+	4.0×10^{-4}

...terionic
...of NaCl

Distance, nm
9.4
4.4
2.0
1.9
0.94

...re 2-7
...on jumps. The curved arrows show the ...taken by protons in successive jumps ...a hydronium ion to a water molecule. ...uid water the jumps are random in ...in ice they occur along the hydrogen-...d lattice. The last H_2O molecule ...becomes a hydronium ion at the end ...eries of proton jumps.



The Ion Product of Water: The pH Scale

The dissociation of water is an equilibrium process:

$$H_2O \rightleftharpoons H^+ + OH^-$$

for which we can write the equilibrium constant

$$K_{eq} = \frac{[H^+][OH^-]}{[H_2O]}$$

where the brackets indicate concentration in moles per liter. The magnitude of the equilibrium constant at any given temperature can be calculated from conductivity measurements

on pure distilled water. Since the concentration of water in pure water is very high (it is equal to the number of grams of H_2O in a liter divided by the gram molecular weight of water, or 1,000/18 = 55.5 *M*) and since the concentrations of H^+ and OH^- ions are very low in comparison (1×10^{-7} *M* at 25°C), the molar concentration of water is not significantly changed by its very slight ionization. The equilibrium-constant expression may thus be simplified to

$$55.5K_{eq} = [H^+][OH^-]$$

and the term $55.5K_{eq}$ can then be replaced by a lumped constant K_w, called the *ion product* of water,

$$K_w = [H^+][OH^-]$$

The value of K_w at 25°C is 1.0×10^{-14}. In an acid solution, the H^+ concentration is relatively high and the OH^- concentration correspondingly low; in a basic solution, the situation is reversed.

K_w, the ion product of water, is the basis for the *pH scale* (Table 2-5), a means of designating the actual concentration of H^+ (and thus of OH^-) ions in any aqueous solution in the acidity range between 1.0 *M* H^+ and 1.0 *M* OH^-. The pH scale was devised by the Danish biochemist S. P. L. Sørensen as a means of avoiding cumbersome numbers like 0.0000001 or 1.0×10^{-7} to express the low hydrogen-ion concentrations in biological fluids. He defined the term pH as

$$pH = \log_{10} \frac{1}{[H^+]} = -\log_{10} [H^+]$$

In a precisely neutral solution at 25°C

$$[H^+] = [OH^-] = 1.0 \times 10^{-7}\ M$$

The pH of such a solution is

$$pH = \log \frac{1}{1 \times 10^{-7}} = 7.0$$

The value of 7.0 for the pH of a precisely neutral solution is thus not an arbitrarily chosen figure; it is derived from the absolute value of the ion product of water at 25°C. It is important to note that the higher the pH number, the lower the hydrogen-ion concentration, and vice versa. Note that the pH scale is logarithmic, not arithmetic. To say that two solutions differ in pH by 1 pH unit means only that one solution has 10 times the hydrogen-ion concentration of the other. Table 2-6 lists the pH of some fluids.

Table 2-5 The pH scale

$[H^+]$, *M*	pH	$[OH^-]$, *M*
1.0	0	10^{-14}
0.1	1	10^{-13}
0.01	2	10^{-12}
0.001	3	10^{-11}
0.0001	4	10^{-10}
0.00001	5	10^{-9}
10^{-6}	6	10^{-8}
10^{-7}	7	10^{-7}
10^{-8}	8	10^{-6}
10^{-9}	9	10^{-5}
10^{-10}	10	10^{-4}
10^{-11}	11	0.001
10^{-12}	12	0.01
10^{-13}	13	0.1
10^{-14}	14	1.0

Table 2-6 pH of some fluids

Fluid	pH
Seawater (varies)	7.5
Blood plasma	7.4
Interstitial fluid	7.4
Intracellular fluids	
Muscle	6.1
Liver	6.9
Gastric juice	1.2–3.0
Pancreatic juice	7.8–8.0
Saliva	6.35–6.85
Cow's milk	6.6
Urine	5–8
Tomato juice	4.3
Grapefruit juice	3.2
Soft drink (cola)	2.8
Lemon juice	2.3

Measurement of pH

Measurement of pH is one of the most common and useful analytical procedures in biochemistry since the pH deter-

mines many important aspects of the structure and activity of biological macromolecules and thus of the behavior of cells and organisms. The primary standard for measurement of hydrogen-ion concentration (and thus of pH) is the *hydrogen electrode*, a specially treated platinum electrode immersed in the solution whose pH is to be measured. The solution is in equilibrium with gaseous hydrogen at a known pressure and temperature. The electromotive force at the electrode responds to the equilibrium

$$H_2 \rightleftharpoons 2H^+ + 2e^-$$

The potential difference between the hydrogen electrode and a reference electrode of known emf, e.g., a calomel electrode, is measured and used to calculate the hydrogen-ion concentration.

The hydrogen electrode proved too cumbersome for general use and has been replaced by the *glass electrode*, which responds directly to hydrogen-ion concentration in the absence of hydrogen gas. The response of the glass electrode must be calibrated against buffers of precisely known pH. Another way of measuring pH is to use acid-base indicators (see below).

Acids and Bases

The most general and comprehensive definitions of acids and bases, applicable to both nonaqueous and aqueous systems, are those of G. N. Lewis. A Lewis acid is a potential *electron-pair acceptor*, and a Lewis base a potential *electron-pair donor*. However, the formalism introduced by J. N. Brönsted and T. M. Lowry is more widely used in describing acid-base reactions in dilute aqueous systems. According to the Brönsted-Lowry concepts, an acid is a *proton donor* and a base is a *proton acceptor* (Figure 2-8). An acid-base reaction always involves a *conjugate acid-base pair*, made up of a proton donor and the corresponding proton acceptor. For example, acetic acid (CH_3COOH) is a proton donor, and the acetate anion (CH_3COO^-) is the corresponding proton acceptor; together they constitute a conjugate acid-base pair.

The equation for the dissociation or ionization of an acid (HA) in dilute aqueous solution involves the transfer of a proton from the acid to water, which itself can act as a proton acceptor to yield the acid H_3O^+:

$$HA + H_2O \rightleftharpoons H_3O^+ + A^-$$

Each conjugate base has a characteristic affinity for a proton relative to the proton affinity of OH^-. Acids that have only a slight tendency to give up protons to water are *weak acids*; acids that readily give up their protons are *strong acids*. The tendency of any given acid to dissociate is given by its *dissociation constant* at a given temperature

$$K = \frac{[H^+][A^-]}{[HA][H_2O]}$$

cale

[OH⁻], M
10^{-14}
10^{-13}
10^{-12}
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
0.001
0.01
0.1
1.0

igure 2-8
me conjugate acid-base pairs.

Proton donor		Proton acceptor
CH_3COOH	$\rightleftharpoons$	$H^+ + CH_3COO^-$
$H_2PO_4^-$	$\rightleftharpoons$	$H^+ + HPO_4^{2-}$
NH_4^+	$\rightleftharpoons$	$H^+ + NH_3$
HOH	$\rightleftharpoons$	$H^+ + OH^-$

ome fluids

pH
7.5
7.4
7.4
6.1
6.9
1.2–3.0
7.8–8.0
6.35–6.85
6.6
5–8
4.3
3.2
2.8
2.3

where the brackets indicate concentrations in moles per liter. It is conventional to simplify this expression by eliminating the water required for hydration of the proton:

$$K = \frac{[H^+][A^-]}{[HA]}$$

It is also the convention in biochemistry to employ dissociation constants based on the analytically measured concentrations of reactants and products under a given set of experimental conditions, i.e., at a given total concentration and ionic strength and with other solutes specified. Such a constant, called an *apparent* or *concentration dissociation constant*, is designated K' to distinguish it from the *true* or *thermodynamic dissociation constant* K employed by the physical chemist, which is corrected for deviation of the system from ideal behavior caused by such factors as concentration and ionic strength.

The apparent dissociation constants of some acids and bases are given in Table 2-7. Note that in the Brönsted-Lowry formalism, acids and bases are treated alike, i.e., solely in terms of the tendency of protons to dissociate from the proton-donor species. (So-called basic dissociation constants, such as K_b for the dissociation reaction $NH_4OH \rightleftharpoons NH_4^+ + OH^-$, are not employed. In fact, in the Brönsted-Lowry formalism, NH_4OH is neither an acid nor a base.) Table 2-7 also gives values for the expression pK', which is a logarithmic transformation of K', just as the term pH is a logarithmic transformation of $[H^+]$:

$$pK' = \log \frac{1}{K'} = -\log K'$$

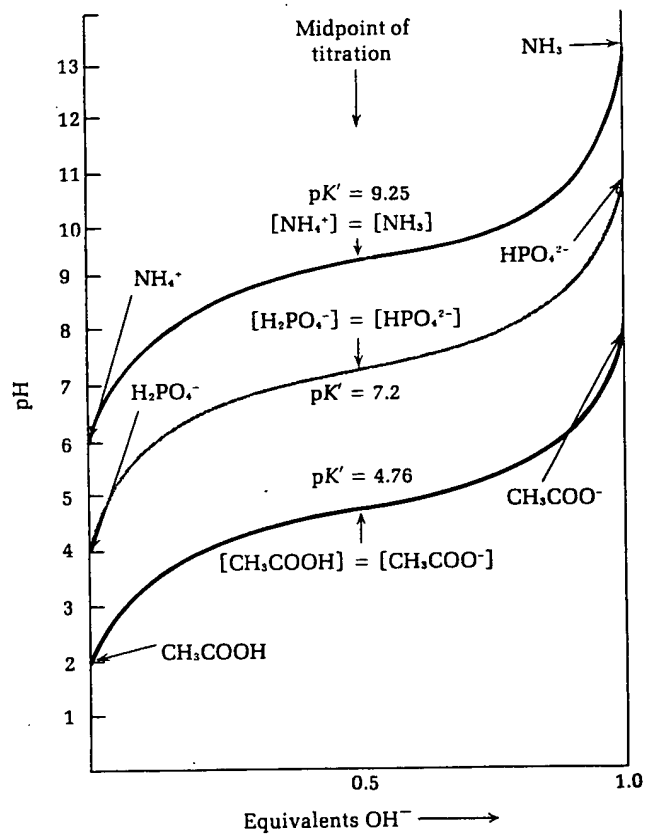
The pK' values are less cumbersome to handle than K' values, just as the pH numbers are less cumbersome than actual hydrogen-ion molarities. Strong acids have low pK' values

Table 2-7 Apparent dissociation constant and pK' of some acids (25°C)

Acid (proton donor)	K', M	pK'
HCOOH	1.78×10^{-4}	3.75
CH_3COOH	1.74×10^{-5}	4.76
CH_3CH_2COOH (propionic acid)	1.35×10^{-5}	4.87
$CH_3CHOHCOOH$ (lactic acid)	1.38×10^{-4}	3.86
$COOHCH_2CH_2COOH$ (succinic acid)	6.16×10^{-5}	4.21
$COOHCH_2CH_2COO^-$	2.34×10^{-6}	5.63
H_3PO_4	7.25×10^{-3}	2.14
$H_2PO_4^-$	6.31×10^{-8}	7.20
HPO_4^{2-}	3.98×10^{-13}	12.4
H_2CO_3	1.70×10^{-4}	3.77
HCO_3^-	6.31×10^{-11}	10.2
NH_4^+	5.62×10^{-10}	9.25
$CH_3NH_3^+$	2.46×10^{-11}	10.6

and strong bases have high pK′ values. Note that water itself may be considered to be a very weak acid of pK′ about 14; its conjugate base, the hydroxide ion, is obviously a very strong base with a high affinity for a proton.

Figure 2-9 shows the titration curves of some weak acids titrated with sodium hydroxide. The pH resulting after each increment of NaOH is plotted against the equivalents of OH^- added. The shapes of such titration curves are very similar from one acid to another; the important difference is that the curves are displaced vertically along the pH scale. The pH intercept at the midpoint of the titration is numerically equal



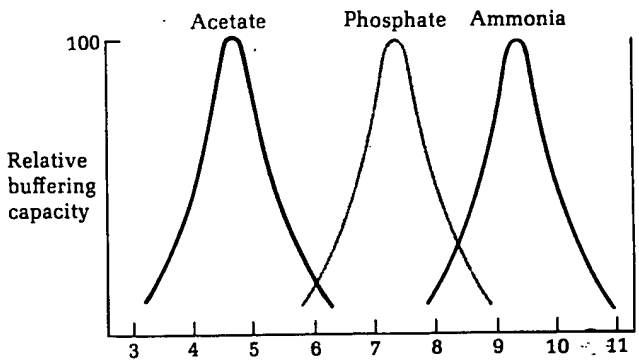


Figure 2-9
(Right) *Acid-base titration curves of some acids, showing the major ionic species at the beginning, midpoint, and end of the titration.* **(Below)** *The relative buffering power of these acids plotted against* pH. *Maximum buffering power is given at* pH = pK′, *at which there is minimum change in* pH *following addition of a given increment of acid or base.*

to the pK′ of the acid titrated. At the midpoint, equimolar concentrations of proton-donor (HA) and proton-acceptor species (A^-) of the acid are present. In fact, the pK′ of an acid can be calculated from the pH at any point on the titration curve of an acid if the concentrations of the proton-donor and proton-acceptor species at this point are known. The shape of the titration curve can be expressed by the *Henderson-Hasselbalch equation*, which is a logarithmic transformation of the expression for the dissociation constant. It is derived as follows:

$$K' = \frac{[H^+][A^-]}{[HA]}$$

Solve for H^+:

$$H^+ = K' \frac{[HA]}{[A^-]}$$

Take the negative logarithm of both sides:

$$-\log[H^+] = -\log K' - \log \frac{[HA]}{[A^-]}$$

Substitute pH for $-\log [H^+]$ and pK′ for $-\log K'$:

$$pH = pK' - \log \frac{[HA]}{[A^-]}$$

If we now change signs, we obtain the Henderson-Hasselbalch equation:

$$pH = pK' + \log \frac{[A^-]}{[HA]}$$

which in more general form is

$$pH = pK' + \log \frac{[\text{proton acceptor}]}{[\text{proton donor}]}$$

This equation makes it possible to calculate the pK′ of any acid from the molar ratio of proton-donor and proton-acceptor species at a given pH, to calculate the pH of a conjugate acid-base pair of a given pK′ and a given molar ratio, and to calculate the molar ratio of proton donor and proton acceptor given the pH and pK′. Note that when the concentrations of proton donor and proton acceptor are equal, the observed pH is numerically equal to the pK′. The Henderson-Hasselbalch equation is fundamental to quantitative treatment of all acid-base equilibria in biological systems.

Acid-Base Indicators

The pH of a solution can be determined by using indicator dyes, most of which are weak acids (designated HInd). Such an indicator dissociates according to the equilibrium

$$HInd \rightleftharpoons H^+ + Ind^-$$